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Infuence of Polymethylene Chain Length and Choline Head Cyclization on Dicholine Esters Hydrolysis by Human Butyrylcholinesterase: An Nmr Approach.

Debouzy Jean-Claude	CLINATEC, centre de recherche biomédicale Edmond J. Safra, commissariat à l'énergie atomique et aux
Nugue Guillaume	IRBA, Institut de Recherches Biomédicales du Service de Santé des Armées, 91223 Brétigny, France.
Selek Laurent	UFR de medicine, Université Joseph Fourier, 38000 Grenoble
Crouzier David	CLINATEC, centre de recherche biomédicale Edmond J. Safra, commissariat à l'énergie atomique et aux

ABSTRACT

The butyrilcholinesterase-induced enzymatic hydrolysis of a dicholine ester derivative (A4), bearing a methyl piperidine head group instead of a choline was studied by 1H-NMR spectroscopy. The various enzyme/substrate molar ratios and concentrations allowed to determine an estimation of K'm of 0,1mM. A4 hydrolysis time course was compared to dicholine derivatives with either a similar acyl chain (4 carbons for DCH4), or similar overall length (DCH8, with a 8 carbon chain). Although a similar initial kinetic slope was found for the three molecules, later evolution was found very close for DCH8 and A4, while slower and more incomplete for A4. These results are discussed in terms of overall physicochemical properties and global hindrance.

KEYWORDS :Choline; Polymethylene Chain; Hydrolysis; Butyrylcholinesterase.

Introduction

Succinylcholin (dicholine ester of succinic acid, DChs) is a molecule with brief lasting myorelaxant properties and a fast hydrolysis in biological medium (Evans, Gray, Lehmann, & Silk, 1952). This property results in a short time clinical effect related with its rapid degradation by plasma butyrylcholinesterase (BuChE) explains its wide use in anaesthesiology (Bevan, 1997; Whittaker & Wijesundera, 1952).

Among many studies on DChs and its metabolites (Dhar et al., 1996; Glick, 1941), a hydrolysis mechanism in two phases has only been recently shown by thin-layer chromatography analysis of BuChE induced hydrolysis. The first step has been identified as the first ether bond leakage in DChs leading to the formation of a monoester and free choline. The monoester is secondly hydrolized into dicarboxylic acid and another choline. The reaction mechanism confirmed by previous studies strongly suggested an affinity difference of the BuChE for DCHS or ester monocholine (MChs)(Whittaker & Wijesundera, 1952).

Besides, the numerous studies dealt with the role of length chain variation between the two ester groups of the dicarboxylic acid (2 to 10) or the serum species (human, horse,...) (Klupp & Stumpf, 1953), gave no definitive conclusion. Moreover, they could not distinguish the two steps mentioned above. For instance, by using length acids range from 2 to 10 methylene, Grigorian's group observed a high disparity in Km and Vmax, with variation of Km and Vmax values increase about 1000 times lower and decrease about 2 times respectively for DChs and MCHS (Grigoryan, Halebyan, Lefebvre, Brasme, & Masson, 2008)a method that allowed monitoring the dicholine substrates, the monocholine intermediates, the dicarboxylic acid and choline products. It was shown that hydrolysis of adipyldicholine involves two consecutive steps, dicholine ester hydrolysis followed by relatively slow monocholine ester hydrolysis. However, sebacyldicholine was hydrolyzed at both choline ester sites, though hydrolysis of dicholine was faster than hydrolysis of monocholine. Sebacyldicholine was completely converted to sebacic acid and choline within 90 min, whereas only 15% of the adipyldicholine was converted to adipic acid in this time. Molecular modeling indicated that these dicholine esters can bind to butyrylcholinesterase in two energetically equivalent alternative conformations that may theoretically lead to hydrolysis. The long chain dicholine ester makes closer contact than the short chain ester between one of its carbonyl carbons and the catalytic Ser198, thus explaining why long-chain dicholine esters are hydrolyzed more rapidly by butyrylcholinesterase.","DOI":"10.1016/j.bbapap.2008.08.005","ISSN":"1570-9639","journalAbbreviation":"Biochimica et Biophysica Acta (BBA.

In contrast to classical techniques (than layers chromatography or release of CO measurement) insensitive, Grigoryan *et al*. combined mass spectrometry (MS) and molecular modelling to demonstrate the association between the BuChE and esters. The long chain dicholine (sebacyldicholine - n=8) was a better substrate than the short chain dicholine (adipyldicholine - n=4). Hence, MS methods enabled to simultaneously identify and monitor in the same spectrum the dicholine esters, monoesters, dicarboxylic acids and choline (Grigoryan et al., 2008)a method that allowed monitoring the dicholine substrates, the monocholine intermediates, the dicarboxylic acid and choline products. It was shown that hydrolysis of adipyldicholine involves two consecutive steps, dicholine ester hydrolysis followed by relatively slow monocholine ester hydrolysis. However, sebacyldicholine was hydrolyzed at both choline ester sites, though hydrolysis of dicholine was faster than hydrolysis of monocholine. Sebacyldicholine was completely converted to sebacic acid and choline within 90 min, whereas only 15% of the adipyldicholine was converted to adipic acid in this time. Molecular modeling indicated that these dicholine esters can bind to butyrylcholinesterase in two energetically equivalent alternative conformations that may theoretically lead to hydrolysis. The long chain dicholine ester makes closer contact than the short chain ester between one of its carbonyl carbons and the catalytic Ser198, thus explaining why long-chain dicholine esters are hydrolyzed more rapidly by butyrylcholinesterase.","DOI":"10.1016/j.bbapap.2008.08.005","ISSN":"1570-9639","journalAbbreviation":"Biochimica et Biophysica Acta (BBA.

The objective of this paper is to give an approach on the role of steric hindrance of the head, using a head-group cyclized derivative methyl piperidine (A4) instead of choline, keeping a total length close to DCH8. ^{1}H-NMR was used, both for A4 structure determination and to compare under similar conditions the results obtained with the derivatives described by Grigoryan (Grigoryan et al., 2008)a method that allowed monitoring the dicholine substrates, the monocholine intermediates, the dicarboxylic acid and choline products. It was shown that hydrolysis of adipyldicholine involves two consecutive steps, dicholine ester hydrolysis followed by relatively slow monocholine ester hydrolysis. However, sebacyldicholine was hydrolyzed at both choline ester sites, though hydrolysis of dicholine was faster

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Materials and methods

Chemicals and enzymes

Dicholine esters of adipic (DCH4) and sebacic (DCH8) and bis derivative (ethyl 2,2 'piperidyil N-N-methyl) adipic ester (A4) were synthesized at the Institute of Fine Organic Chemistry (Institute of Fine Organic Chemistry (TBI), Yerevan, Armenia) (Figure 1). Their chemical structure has been monitored by conventional NMR methods, predictive models and by mass spectrometry. Other reagents were of analytical grade. The butyrilcholinesterase (BuChE) highly purified and conditioned at 0.5 mg /ml in 0.1M phosphate buffer (pH 7.0) was kindly provided by Prof P.Masson and O. Lockridge (UNMC, Eppley Institute for Cancer Research, Omaha, NE, USA). A solution at 0.5 mg/ml, 0.5 g/l gives a molar concentration of 1.42 µM. Correspondence between the units (U) and molarity (M) assumes that the enzyme solution is 100% active (all functional active sites, no inactive forms). Under these conditions, it was found that for activity measurements with butyrylthiocholine (1 mM) at pH 8.0 and 25°C , this correspondence was 1 mg = 700 units, that is to say that 10 µl of a solution 7 U/ml corresponding to 0.5 nM in an NMR tube 500 µl.

Nuclear magnetic resonance (NMR)

Spectra were recorded on a Bruker Avance 400 NMR spectrometer operating at 9.4T (Larmor frequency of the proton at 400 MHz). The temperature was regulated to 0.2°C by a Bruker BVT2000 unit. Pre-saturation sequence of the water resonance was used. The spectra were collected on data 32K for a spectral window of 10 ppm. Structures representation and formulas have been made from the ChemCAD and ACDLab software.

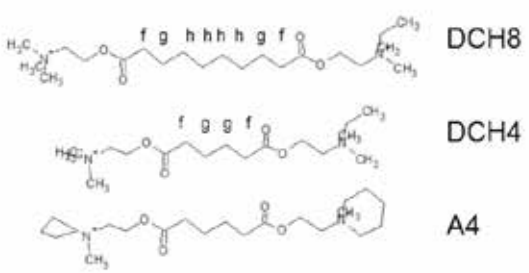

Figure 1: general structures of dicholine esters and acidic moiety proton nomenclature ; from top to bottom, DCH4, DCH8, A4.

Results

A4 Enzymatic hydrolysis.

A4 structuring in aqueous solution.

Figure 2B shows the NMR spectrum of A4 in aqueous solution. Although the groups are easily identifiable (αβY and cycle resonances abc, length fg), the linewidth of a typical resonance (3.5Hz) was not compatible with a real solution, suggesting the formation of supramolecular associations, micelles or aggregates. This widths corresponded with a T2 values of approximatively 100 ms, and with T1 values close to 200 ms, allows using Solomon-Bloemenberg equations:

$$\mathbf{R1 = 1/T1 = A.\tau_c.[(1/(1 + \omega^2\tau_c^2) + 4/(1 + 4\omega^2\tau_c^2)]}$$

$$\mathbf{R2 = 1/T2 = 6A.\tau_c.[4 + 9/(1 + \omega^2\tau_c^2) + 6/(1 + 4\omega^2\tau_c^2)]}$$

With ω=400MHz; A = $\gamma^4(h/2\pi)^2/r^6$; γ=gyromagnetic factor; (h/2π) Planck constant ; r=distance inter spin

To extract τ_c~5ns, with Stokes-Einstein equation:

$\tau c = \eta V/kT$, soit $V = kT.\tau c/\eta$ **(12)**

with η=0.9x10^{-3} P, (N.s/m^2 at 298K), k=1.38 x 10^{-23}J/kg, T=297 K and the volume V (m^3).

By using a spherical approximation, an estimate of the aggregate volume of 3800 Å (diameter=19 Å) was proposed. Considering the maximum length of A4 (Table III) of 18Å, this hypothesis of aggregate formation could be refuted.

Kinetics of hydrolysis of A4.

Figure 2A shows the spectrum recorded 30 minutes after addition of BuChE. For several resonances (i.e. αβY and fg), a second population was detected at high field (table II), whereas neither the linewidths, couplings constants or the position of chemical shift of the initial population exhibit any significant variation. In the absence of effect of temperature or field (not shown in the present work), the existence of a true reaction (bio) chemical was ascertained, and exchanging mechanisms or simple binding processes refuted.

It is noteworthy that the chemical shift differences between initial positions and lines of new formed species are all the more significant as the corresponding group is close to the maximum hydrolysis site for αβ and f (see table II). Conversely chemical shift variations are almost negligible or absent for the other resonances, in agreement with the upfield shield related with the loss of connections via the ester bond (electronegativity of carboxylic O).

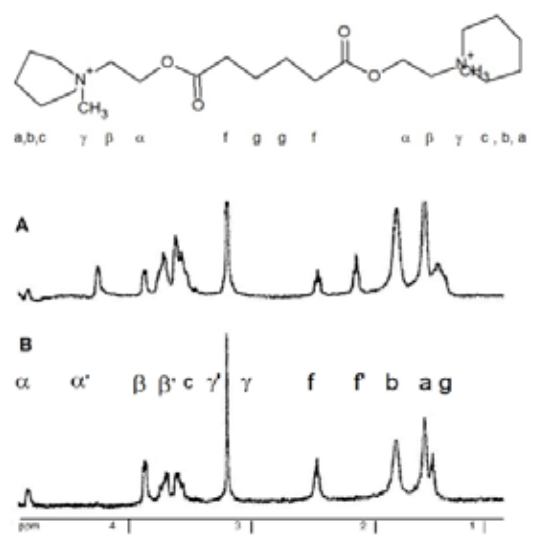

Figure 2: top trace: proton nomenclature; bottom A) 1H-NMR spectrum of A4 0,5mM, 298K, D2O phosphate buffer, pH8, alone and B) in the presence of de 10µL BuChE (0,5mg/mL), after a 90 minutes time course.

To access the enzymatic parameters, concentrations and proportions of substrates and BuChE were varied on a scale of 0.1-1mM (A4) and 0.1- 0.5Nm (BuChE) (Figure 3). Velocities depending on the concentration A4 allowed to propose an estimation of K'm = 0.1 mM and to draw Vmax from the regression line (Figure 4) :

1/S=220 [A4]+22.18,

with [A4] in mM.

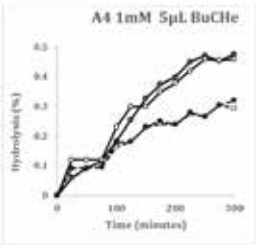

Figure 3: relative hydrolysis production observed on α (●), f([□), β (-●-) and g (-○-) width.

Note here that neither the curves built from hydrolysis curves or degradation product setup allowed to clearly identify the four species involved in the biochemical course: unhydrolysed substrates (A4 and DCHS), mono hydrolysed (1 group released on A4 and MCHS), dicarboxylic acids, and released groups (piperidine or choline). For example, the initial peak α is an esterified group again whatever its origin (unhydrolysed of A4 mono substituted), whereas the peak α' appeared during kinetic is identical for the fully hydrolysed form (2 groups), or mono-hydrolysed of A4.

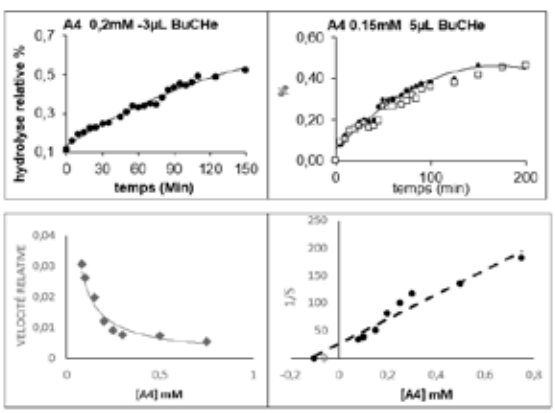

Figure 4: top traces of relative A4 hydrolysis for two different enzyme/substrate ratios, built from f (•) or g(•) width. Bottom traces: degradation velocity of A4 as a function of BuChE concentration and affinity determination from the reverse slope the left trace.

Comparison with the dicholin esters, DCH4, DCH8.

The hydrolysis DCH4, DCH8 and A4 substrate curves were recorded under the same conditions previous used here (BuChE 1 and 0.5 nM, Figure 5). Whereas it was not possible, as in conventional methods (Ginzel, Klupp, & Werner, 1951), to discuss hydrolysis courses on these curves, both initial slope and evolution could be observed followed by peak integration on NMR spectra.

Thus, on the slopes established the first 20 minutes, both DCH derivatives exhibit higher values than A4, or A4 (0.0021 / min) for DCH8 (0.005 / min), and for DCH4 (0.0071 / min). The evolution in time well fitted a mono exponential dependence for DCH4,

$y = 0{,}67\exp(-0{,}003t)$

while not clearly identifiable for DCH8. Rather a linear evolution was observed for A4 (slope of 0.0015/min). This feature was also in agreement with A4 further and late evolution reaching that of DCH8 hydrolysis after 100 min (0.0015/min). Conversely, DCH4 traces turned to a quasi-steady phase (0.0003/min). Final evolution (300min) showed similar yields for DCH8 and A4 with 49.5% and 52% of hydrolysis respectively, whereas this rate was only 38% for DCH4.

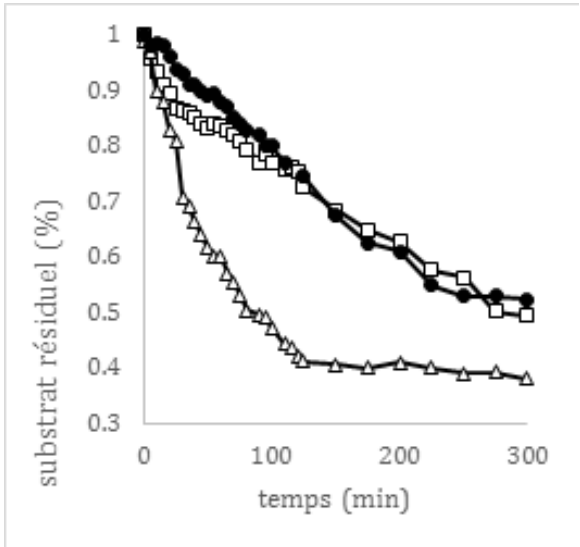

Figure 5: Time course of relative hydrolysis (%) for A4 (•), DCH4(Δ) and DCH8 (□) for a 1mM initial substrate concentration and 10µL (0,5nM) BuChE.

Discussion

The objective of this work was to characterize and evaluate a cyclized piperidine derivative of reference dicholine structures to obtain structural, conformational or physico-chemical arguments to lead further derivatives synthesis. Hence, A4 both similarities and differences were identified with each of the two structures (DCH4 and DCH8). Thus, cyclization of choline in methylpiperidine while resulting in an increased volume at this level, also provides an increase of the chain length, finally close to that of DBCH8 (see table II). Conversely, the flexibility obtained in C8 chain as DCH8, does not exist for A4: this central part, between the two esters bonds, is identical to DCH4.

Table I: General data for A4, DCH4 and DCH8.

molecule	MW	LogP	distance N-C	distance CO/CO	longueur totale	accessibilité	Δv/2	tête polaire
DBCH4	318,45	-5,03	4,8	6,4	17,8	3 CH3	0,5Hz	choline
DBCH8	388	-3,34	4,8	11	23,1	3CH3	0,5Hz	choline
A4	354,6	-1,29	4,8	6,3	17,8	plan de groupe	3_5Hz	piperidine
ACHE	146,2	-3,9	4	_	7,7	totale	0,2Hz	choline

A4 solubility: According with their good solubility in water, both DCH4 and DCH8 give well-resolved spectra while relatively broader line width are measured on A4 (3.5-4Hz, Table II) which seems not in agreement with a true solution, rather with supramolecular assemblies. This hypothesis was refuted from relaxation time measurements since the apparent calculated diameter was very close to the length of the molecule (18-20A). The minor structure variations realized can then be related with the significant physic chemical properties observed, e.g. solubility. This is a common feature, as observed for instance for natural cyclodextrins: passage 6 to 7 subunits sufficient to bring down the solubility in water of 125 mM (α-cyclodextrin) to 19 mM β-cyclodextrin) (Szejtli, 1982).

Table II: Chemical shifts (Δ, ppm) and chemical shift variations (Δδ, ppm) after hydrolysis by BuChE. High field variations are quoted negatively.

molecule	ppm	a	b	c	f	g	h	α	β	γ
DBCH4	δ	_	_	_	2,44	1,61	_	4,69	3,63	3,3
	Δδ	_	_	_	-0,1	x	_	-0,4	-0,3	-0,1
DBCH8	δ	_	_	_	2,51	1,6	1,26	4,71	3,6	
	Δδ	_	_	_	-0,3	-0,15	0	-0,4	-0,2	-0,1
A4	δ	A,75	1,88	3,5(m)	2,45	1,65	_	4,52	3,62	_
	Δδ	x	x	0	-0,3	-0,1	_	-0,4	-0,25	ns

*A4 Comparison with DCH4 and DCH8***.**

Grigoryan proposed a 2-phases hydrolysis for DCH4: under this hypothesis, the initial step should correspond to the present results, since the adipic acid (AA) production does not exist, and that produc-

tion MCH4 only occurs. At intermediate times, this monoester production and appearance of AA simultaneously exist, the later being exclusively present at the end of the kinetics by competition between MC4 degradation and AA production, which finally does not reach 50 %.

For DCH8, this trend is less observable due to the early sebacic acid (SA) production and the rapid DCH8 deterioration in MCH8. These competitions are not identifiable by NMR, the overall kinetics looking slower than DCH4 for early times of evolution. Conversely, later hydrolysis works better, with a final production over 50% hydrolysis at the end of the experiment.

It is worth to note here that time course used in Grigoryan's works were much shorter than in the present results due to lower enzyme concentration conditions (0.5 nM versus 3nM for Grigoryan Grigoryan), precisely chosen to slow kinetic course and make the experiment recordable NMR. For the same reason, a double concentration of substrate (1mM) was also used here (Grigoryan et al., 2008)a method that allowed monitoring the dicholine substrates, the monocholine intermediates, the dicarboxylic acid and choline products. It was shown that hydrolysis of adipyldicholine involves two consecutive steps, dicholine ester hydrolysis followed by relatively slow monocholine ester hydrolysis. However, sebacyldicholine was hydrolyzed at both choline ester sites, though hydrolysis of dicholine was faster than hydrolysis of monocholine. Sebacyldicholine was completely converted to sebacic acid and choline within 90 min, whereas only 15% of the adipyldicholine was converted to adipic acid in this time. Molecular modeling indicated that these dicholine esters can bind to butyrylcholinesterase in two energetically equivalent alternative conformations that may theoretically lead to hydrolysis. The long chain dicholine ester makes closer contact than the short chain ester between one of its carbonyl carbons and the catalytic Ser198, thus explaining why long-chain dicholine esters are hydrolyzed more rapidly by butyrylcholinesterase."","DOI":"10.1016/j.bbapap.2008.08.005","ISSN":"1570-9639","journalAbbreviation":"Biochimica et Biophysica Acta (BBA.

A4 hydrolysis appears linear from the beginning, with a similar slope as that of DCH8 hydrolysis at late times, and an identical yield (50%). Considering DCH8 kinetics, this can be explained by the immediate or very early involvement of A4 monoester with an even more hydrolysis of A4 monoester in A. The shorter period would then imposing the apparent course the overall kinetics (this is also conventionally used, including nuclear industry, to produce short-lived derivatives from long radioactive period precursors (Tubiana, Dutreix, & Pierquin, 1996).

In fact, the steric hindrance conferred by the piperidine ring coupled with more amphiphilic properties results in a greater accessibility to the piperidine building block, which finally could explain the setup of a two step A4 hydrolysis. The confirmation will need to perform the same experiments using mass spectroscopy on A4, DCH4 DCH8 and under the conditions of this study.

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REFERENCES

Bevan, D. R. (1997). Neuromuscular blocking drugs: onset and intubation. Journal of Clinical Anesthesia, 9(6 Suppl), 36S–39S. Dhar, N. C., Maehr, R. B., Masterson, L. A., Midgley, J. M., Stenlake, J. B., & Wastila, W. B. (1996). Approaches to short-acting neuromuscular blocking agents: nonsymmetrical bis-tetrahydroisoquinolinium mono- and diesters. Journal of Medicinal Chemistry, 39(2), 556–561. http://doi.org/10.1021/jm950477z Evans, F. T., Gray, P. W. S., Lehmann, H., & Silk, E. (1952). Sensitivity to succinylcholine in relation to serum-cholinesterase. Lancet, 1(6721), 1229–1230. Ginzel, K. H., Klupp, H., & Werner, G. (1951). [On the pharmacology of alpha, omega-bis-quarternary ammonium compounds. III. Enzymatic splitting of certain aliphatic dicarboxylic acid esters and their potentiation by eserine]. Archives Internationales De Pharmacodynamie Et De Thérapie, 87(3), 351–365. Glick, D. (1941). Some Additional Observations on the Specificity of Cholinesterase. Journal of Biological Chemistry, 137(1), 357–362. Grigoryan, H., Halebyan, G., Lefebvre, B., Brasme, B., & Masson, P. (2008). Mechanism of hydrolysis of dicholine esters with long polymethylene chain by human butyrylcholinesterase. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1784(11), 1818–1824. http://doi.org/10.1016/j.bbapap.2008.08.005 Klupp, H., & Stumpf, C. (1953). [Differences in the enzymatic splitting of the di-choline esters of dicarboxylic acids by human, horse and dog sera]. Enzymologia, 16(3), 189–192. Szejtli, J. (1982). Cyclodextrins and their inclusion complexes. Akademiai Kiado. Tubiana, M., Dutreix, J., & Pierquin, B. (1996). One century of radiotherapy in France 1896-1996. International Journal of Radiation Oncology, Biology, Physics, 35(2), 227–242. Whittaker, V. P., & Wijesundera, S. (1952). The hydrolysis of succinyldicholine by cholinesterase. Biochemical Journal, 52(3), 475–479.